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PRINCIPAL INVESTIGATOR: Robert F. Mattrey, M.D.

CONTRACTING ORGANIZATION: University of California, San Diego
La Jolla, California 92093-0934

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Robert F. Mattrey, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, San Diego
La Jolla, California 92093-0934

E-Mail: lgehrke@ucsd.edu

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13. ABSTRACT *(Maximum 200 Words)*

Purpose: During the first year, we identified a radiopaque tumor-marking agent and optimized the dose. During the second year we worked on coloring the agent and worked on the second agent for sentinel node detection to improve upon the water-soluble dye.

Scope: We could not color the radiopaque perfluorocarbon (PFC) emulsion but successfully suspended India Ink within it. The optimum dose of the mixture was injected in tumors. We followed the black tract to locate and remove the tumor at 72 hours. The resected specimen was then radiographed. We began the work to accomplish the 3rd goal, the identification of the sentinel node.

Results: The black color guided us to the tumor in all rabbits. The radiopaque agent was visible within the tumor at 72-hours. As we learned that it is difficult to color PFC emulsions, we abandoned the non-radiopaque PFC emulsion approach to locate the sentinel node. We identified a lipid emulsion where Sudan Black is dissolved within the vesicles. During this final year we are testing the blue emulsion and comparing its pharmacokinetics and sentinel node enhancement ability relative to the water-soluble blue dye.

Significance: We have accomplished 2 of the 3 goals to date, marking the tumor with a radiopaque agent for specimen radiography and marking the tract to guide the surgeon to the tumor. We have identified a blue lipid emulsion that should have a longer dwell time in the lymphatics and a greater ability to localize in the sentinel node than the water-soluble dye. Our ultimate goal is to provide accurate non-invasive sentinel node localization to make resection an office procedure that could be done under local anesthesia.

14. SUBJECT TERMS
(1) contrast media; (2) tumor marking; (3) ex vivo proof of specimen; (4) radiopaque perfluorocarbon emulsion; (5) sentinel node marking

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Introduction

Our grant seeks solutions to 2 related problems in breast cancer surgery and imaging: (1) provide the ability to mark non-palpable and non-radiopaque lesions to guide surgery and prove their resection on specimen radiography. (2) Mark and localize the sentinel node. There is an increasing number of non-palpable lesions detected on MRI and ultrasound limiting guidance and the ability to confirm that lesions have been successfully resected. We aimed to accomplish these two goals using perfluorocarbon (PFC) emulsions. The particles of an emulsion, particularly when their content is not water soluble, clear slowly from the injection site. If these particles are colored and radiopaque, they will provide an opportunity to guide the surgeon to the tumor and for prolonged visualization of the injection depot to allow specimen radiography. Because the particles are cleared by lymph, it is possible to color the emulsion and thus prolong visualization of the lymph duct and node compared to the water-soluble dye, Lymphazurin.

Body of Progress Report

Because it was known that PFC emulsions, that can be radiopaque, remain at the injection site for several days and because it was also known that these PFC emulsions are removed by the local lymphatic circulation and accumulate in macrophages in the draining nodes [Wolf GL et al. *Radiology* 1994 191:501-5] we aimed to optimize these emulsions and to color them to achieve the goals outline above. In the first year of this award, we demonstrated the feasibility of marking tumors with the radiopaque PFC emulsion in a rabbit model. In the second year, we refined our technique of marking tumors by tailoring the injection dose to the size of the tumor and site of injection using ultrasound guidance. This allowed us to limit the amount of contrast extravasation as well as optimize the enhancement of the tumor site. During the third year, we attempted to color the PFC emulsion and tested whether fluorescent labeling was feasible. Although we suggested in our proposal that we would use fluorescent labels to provide visual guidance, we sought a solution that would allow direct visualization of the dye under ambient light. We suspended India Ink within the radiopaque perfluorocarbon (PFC) emulsion and succeeded to produce a black suspension. We were successful at marking the lesions. We refined the technique to minimize extravasation and to limit the agent to the tumor. We have reported our progress in both an abstract as well as poster presentation at the Department of Defense: Era of Hope Meeting, June 8-12th, 2000 "Marking Mammographically Invisible Tumors for Surgical Guidance and Proof of Resection by Ex-vivo Radiography" (see Appendix). In brief, The India ink colored radiopaque PFC was identified in all lesions at 72 hours that aided in tumor localization and excision at necropsy. Specimen radiographs taken following tumor resection showed absence of radiopaque material in the leg and the presence of the radiopaque material in tumor confirming the resection of the marked tumor. Interestingly, the ipsilateral popliteal node enhanced on CT in 83% of rabbits relative to the control leg allowing their easy detection. This demonstrates that particulates injected in the tumor with minimal or no extravasation can accumulate in the draining nodes. Because PFC emulsions are also visible on ultrasound, sonographic enhancement of the popliteal nodes was also observed.

The second major aim of the award was to help localize the sentinel lymph node by improving upon the water-soluble dye. The major weakness of the water-soluble dye is its rapid and unpredictable transit through the lymphatic system following subcutaneous injection. The goal was to produce a visible particulate emulsion. We had successfully enhanced lymphnodes for CT scanning and for ultrasound imaging using an indirect lymphography technique. Lymphnodes accumulate particles injected in the subcutaneous space by capturing macrophages

that had ingested the particles from the injection site or by the resident macrophages ingesting the particles as the latter traverse the lymphatics. Since the goal is to provide visual guidance to the sentinel node, we investigated PFC emulsion. We knew that PFC liquid is hydrophobic but we discovered that PFCs were also sufficiently lipophobic that lipid soluble dyes could not be dissolved within the particle. We then sought a non-PFC emulsion approach and identified an experimental formulation that could serve this function. We dissolved a lipophilic dye (Sudan Black) in a triglyceride emulsion and proceeded to test it and compare it to the water-soluble dye, Lymphazurin.

We performed a preliminary study using 28 rabbits to determine the dose of contrast and time points. We started with 0.04% w/v Sudan black with 20% v/v TG emulsion. We found that to quantify the amount of Sudan Black in the lymph nodes with a spectrometer, we needed a higher concentration of Sudan Black. Based on these findings we decided to increase the amount of Sudan Black from 0.04% w/v to 0.4% w/v. We planned a new experiment to determine the pharmacokinetics of the blue emulsion compared to a water-soluble dye (Lymphazurin).

We injected the new TG-Sudan Black Emulsion (0.4% w/v Sudan Black) into one hind footpad and Lymphazurin (0.5 ml each) into the other footpad of anesthetized rabbits, then euthenized the rabbits at various time points (5 min., 30 min., 1 hr., 2 hrs., 3 hrs., 6 hrs., 12 hrs., and 24 hrs.) and removed their popliteal and iliac lymph nodes. We ran into problems with the new blue emulsion during the first experiment. We noticed that the 12 and 24-hour rabbits had fever and swollen feet. Therefore, we repeated rabbits at the 3 hour and 6 hour time points and kept track of their temperatures and watched for foot swelling. The three-hour rabbit developed a temperature but no swelling while the six-hour rabbit developed both. Having ruled out health problems and contamination, we tried different concentrations (0.2, 0.08, 0.04 % w/v) of Sudan Black to see if the new higher concentration was the problem, and also checked our sterile procedures. With the lower concentration batch, the feet swelled less, but the fever remained. Emulsion without Sudan Black caused swelling but no fever.

Although we planned to remove all nodes to quantify the amount of Sudan Black and Lymphazurin, we only did so 8 rabbits. Figure 1 shows the 5-point blue intensity scale used to grade nodes objectively and Figure 2 shows the results from the first group of 8 rabbits. The remainder of the effort went into optimizing the blue emulsion. We believe the most likely reason for the reaction is type of lipid used for the emulsion and a less likely but possible reason is the Sudan Black itself.

Fig.1 The color intensity chart for the scaling.

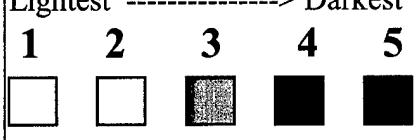
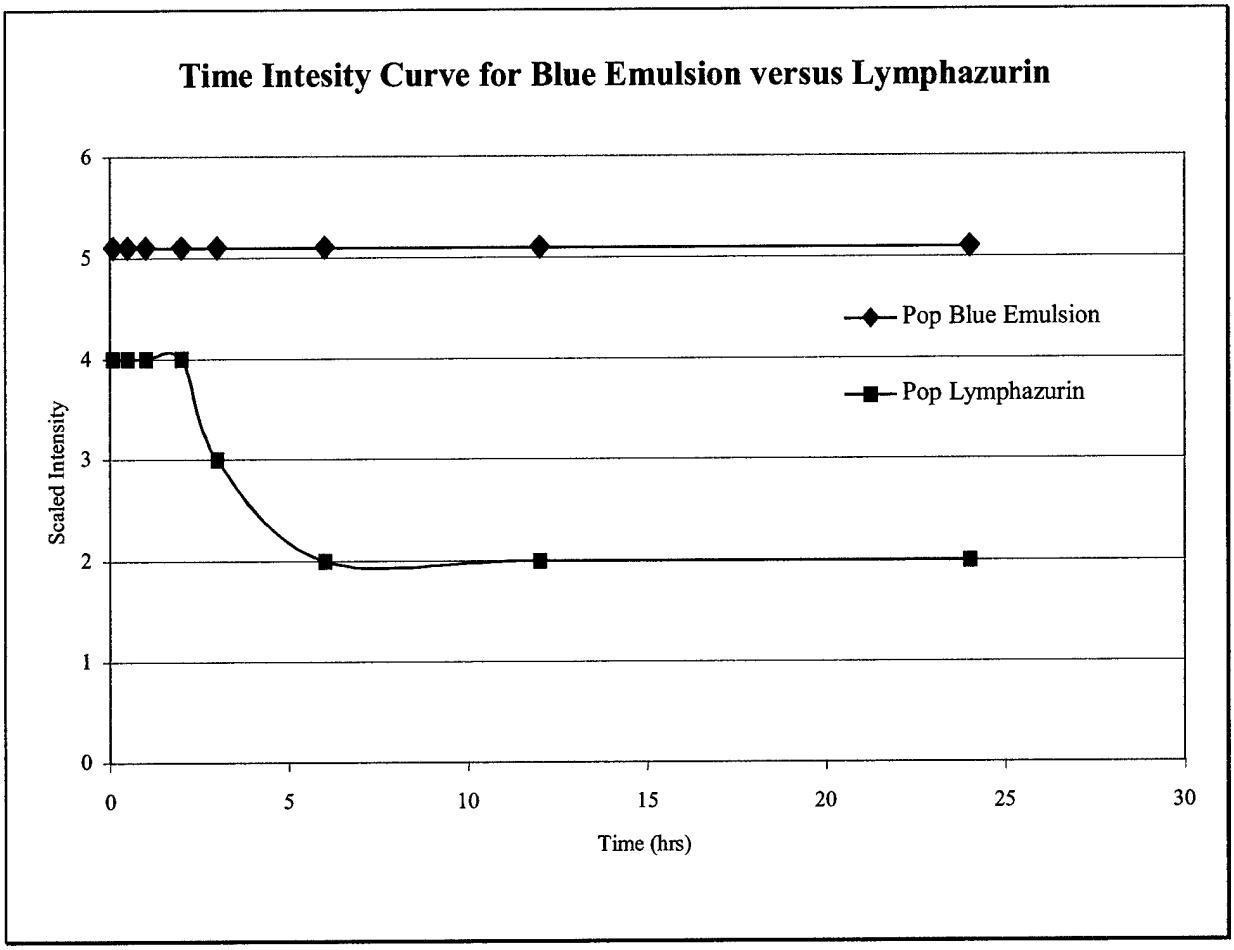


Fig.2 The time intensity curve for the blue emulsion (BE) and water-soluble blue dye, Lymphazurin (Lym). Note that BE in the popliteal nodes (Pop BE) stayed significantly dark at 24 hours, while Lymphazurin in the popliteal nodes (Pop Lym) stayed dark for only 2 hours. Note that the amount of Lymphazurin was significantly high compared to clinical dose. **[I think we should not include iliac data]**



Key Research Accomplishments

⇒ We are able to use ultrasound guidance to inject contrast and mark tumors and visualize the area of injection with X-ray and CT.

⇒ We have determined the variables that promoted extravasation and are able to avoid extravasation.

⇒ We showed by CT that the radiopaque agent was still visible within the tumor by 72 hours after injection.

⇒ We were able to fill the draining node (sentinel node) with radiopaque contrast and image the node by CT after the intra-tumoral injection.

⇒ We are able to quantify contrast washout from the tumor, degree of leakage, and lymphatic accumulation using CT images.

⇒ We were successful at suspending India Ink, a black marker, in the radiopaque emulsion and showed that we can mark the lesion for several days in-vivo and upon resection, visualize the dye within the tumor.

⇒ We have investigated several approaches for sentinel node visualization. Although the triglyceride emulsion with Sudan Black seems promising as it colored the lymph ducts and node for several hours, we have encountered an adverse event with the agent that we could not resolve during the time of the award.

Reportable Outcomes

A manuscript is near completion to report the findings describing tumor marking and node visualization that was already presented and referenced below.

Conclusions

This award allowed us to successfully demonstrate the ability to mark tumors for days, mark the track for tumor resection and confirm on specimen radiography that the marked region was removed. Although we were successful at using a colored particulate agent for marking the lymph duct and node, the agent has side –effects that we could not resolve within the award period.

Bibliography:

1. Pinnell SP, Kono Y, Diranieh L, Mattrey RF. Marking Mammographically Invisible Tumors for Surgical Guidance and Proof of Resection by Ex-vivo Radiography.

Appendix A

MARKING MAMMOGRAPHICALLY INVISIBLE TUMORS FOR SURGICAL GUIDANCE AND PROOF OF RESECTION BY EX-VIVO RADIOGRAPHY

S. P. Pinnell, M.D., Y. Kono M.D., L. Diranieh, and R. F. Mattrey, M.D.

Department of Radiology
University of California, San Diego

Purpose: The confirmation that lesions seen on mammography have been removed is possible with specimen radiography. This option is not possible when tumors are detected by alternate techniques. We identified a radiopaque perfluorocarbon (PFC) emulsion that was successfully tested in a phase I study for indirect lymphography were it was shown that the agent remained at the injection site for several days [1]. Our primary goal was to determine that this agent when injected in the tumor would remain at the injection site to mark the lesion for specimen radiography. There were 3 secondary aims. The first was to determine if coloring the PFC emulsion would allow surgical guidance to the lesion in order to decrease the inconvenience of needle localization and allow pre-operative localization days before surgery. The second was to determine the maximum volume of the agent that could be injected in the tumor without contaminating the surrounding tissues. The third was to determine if the radiopaque PFC particles would reach the draining lymph nodes to allow the potential of identifying the location of the sentinel node for pre-operative sentinel node localization.

Methods/Materials: Vx2 tumor was implanted in one calf of 12 rabbits and both calves in 6 rabbits and allowed to grow for 14 to 22 days. The 12 rabbits with single tumors were used to define the maximum volume that could be injected into the tumor without causing extravasation. To accomplish this, we calculated tumor volume at the time of injection as (4/3) x (Pi) x (A/2) x (B/2) x (C/2); where A, B, and C are the 3 orthogonal diameters of the tumor as measured by ultrasound. Using ultrasound guidance, a needle was inserted into the tumor center and serial radiographs were obtained following the injection of 0.1ml increments of the PFC emulsion (AF1053, Alliance Pharmaceutical Corp. La Jolla, CA). The volume of the agent at which extravasation occurred was recorded. The linear correlation relating extravasation volume and tumor volume was calculated.

For the remaining six rabbits with 2 tumors in each calf, 0.1 ml of India ink was added to 1 ml of PFC emulsion and 90% of the extravasation volume was injected into one of the two tumors based on the equation defined above. The tumor in the other leg served as control. The tumor, popliteal fossa, and periaortic regions were imaged at 1, 24, 48, and 72 hours with x-ray and ultrasound. At 72 hours, the rabbits were imaged with CT immediately post sacrifice using 3mm slice thickness serially from the tumor to mid retroperitoneum. Immediately following CT, the leg was photographed and the tumor was resected, its size was measured, and the location of the emulsion assessed. The resected leg and the tumor were then radiographed.

Results: The radiopaque PFC emulsion was clearly seen on radiographs (Fig. 1). The linear correlation between tumor volume and the volume of PFC that just caused extravasation was 0.13 x tumor volume (cm3) + 0.49 (r=0.77) (Fig. 2). In 5 of the 6 rabbits injected with the optimal dose, minimal to no extravasation occurred immediately after injection. The single tumor

where extravasation occurred was small in size making needle placement difficult. Although the radiopacity of the tumor diminished slightly it was still visible at 72 hours (Fig. 3). The India ink colored PFC was identified in all lesions at 72 hours (Fig. 4) which aided in tumor localization and excision at necropsy. Specimen radiographs taken following tumor resection showed absence of radiopaque material in the leg and the presence of the radiopaque material in tumor confirming the resection of the marked tumor (Fig 3).

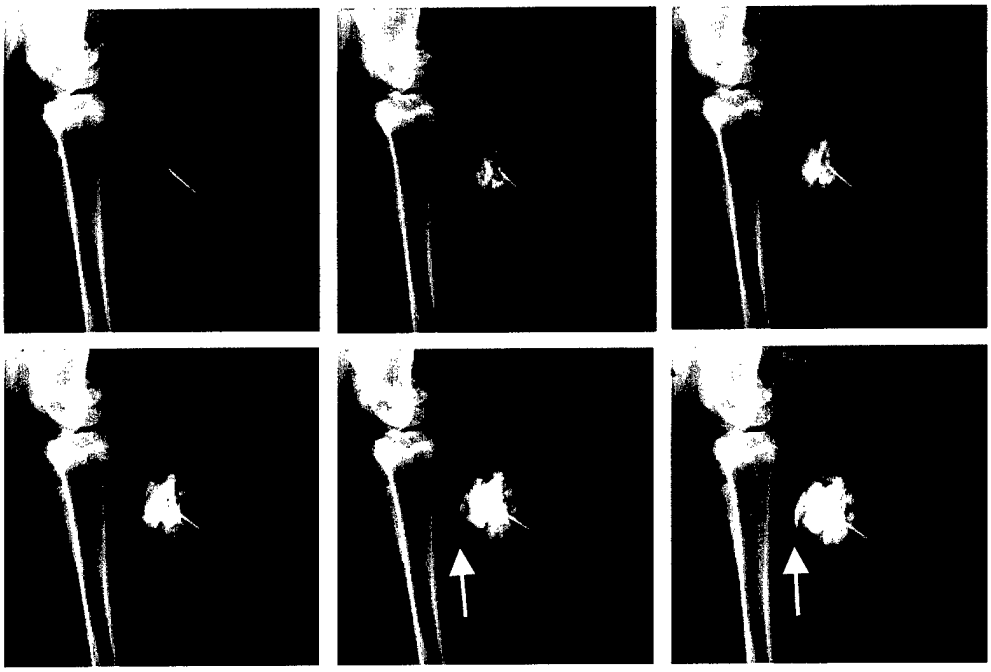
The ipsilateral popliteal node enhanced on CT in 5 of the 6 rabbits by 37.8 ± 17.6 HU relative to the control leg ($p<0.05$) allowing their easy detection (Fig. 5). This demonstrates that particulates injected in the tumor with minimal or no extravasation can accumulate in the draining nodes. However, in 2 of the 6 rabbits which displayed local popliteal lymph node enhancement, the retroperitoneal lymph nodes enhanced by 96 and 97 HU (Fig. 6) indicating that the agent is not limited to the first draining lymph node. Because PFC emulsions are also visible on ultrasound [2], sonographic enhancement of the popliteal nodes was also observed (Fig. 7).

Conclusion: This study showed that the radiopaque PFC emulsion can be colored using India ink. The colored radiopaque emulsion remained at the injection site for at least 72 hours and the addition of India ink aided as a guide to the tumor during resection. It was possible to inject an optimal dose of the agent in this tumor model that did not contaminate the tissues surrounding the tumor. Although the radiopacity in the lesion decreased slightly over 3 days, the agent was still visible at the time of necropsy. Despite a central tumor injection, the particulate contrast agent accumulated in the first node draining the tumor in five of six rabbits and in downstream nodes in 2 of 6 rabbits. Since the agent also has sonographic properties, ultrasound was used to detect local lymph node drainage which may provide pre-operative sentinel lymph node localization in the future.

References:

1. Wolf GL, Rogowska J, Hanna GK, Halpern EF. Percutaneous CT lymphography with perflubron: imaging efficacy in rabbits and monkeys. *Radiology* 1994;191:501-5
2. Wrigley RW, Saunders HB, Lim G, Arellano RA, Mattrey RF: Indirect Ultrasonographic Lymphography with Perflubron Emulsion. RSNA Chicago, *Radiology* 1993; 189:285.

Figure 1: Serial radiographs of the calf following the injection 0.1, 0.3, 0.5, 0.7, 0.9, and 1.1 ml of AF1053 into the tumor. Note that extravasation (arrow) occurred after 0.9 ml and became more apparent after 1.1 ml.



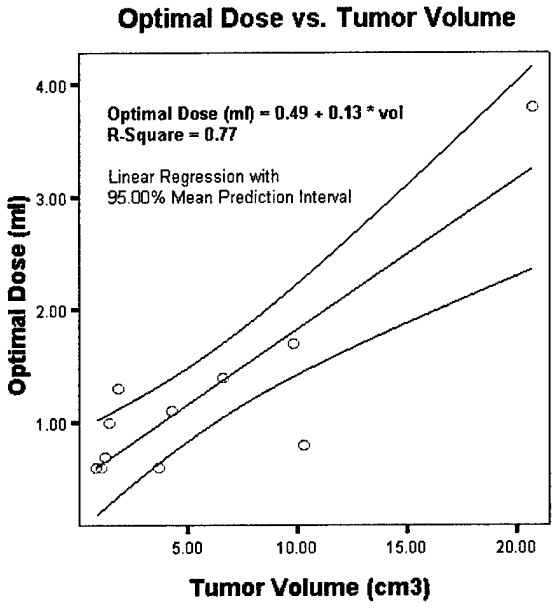


Figure 2: Scatter plot displaying the correlation of emulsion volume that caused extravasation.

Figure 3: Specimen radiograph of the leg and tumor (arrow). Note that the tumor is radiopaque and the tumor site on the leg (arrowhead) has no radiopaque material.

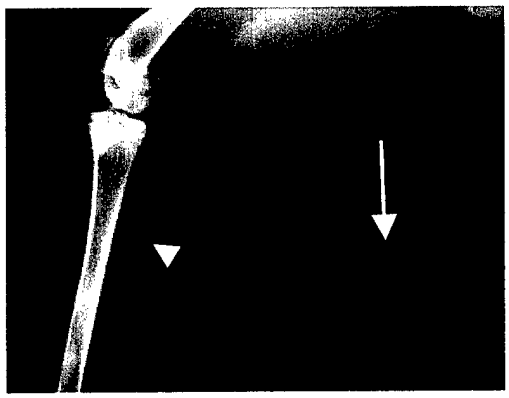


Figure 4: Photograph of leg and resected tumor shown in Figure 3. Note the black dye in the tumor at the time of resection.

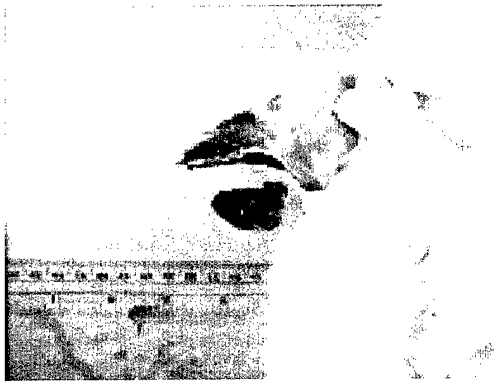


Figure 5: CT of the popliteal region demonstrating enhancement of a portion of the ipsilateral node (arrow).

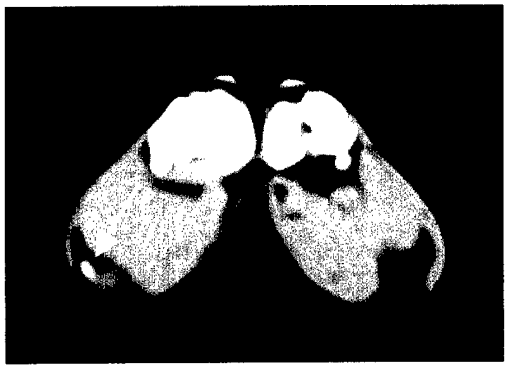


Figure 6: CT of the retroperitoneum demonstrates enhancement of an ipsilateral iliac node (arrow).

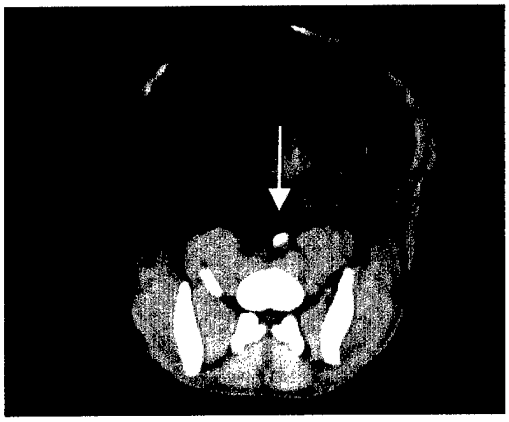


Figure 7: Ultrasound scan of a popliteal node obtained before (a) and 24 hours (b) following the injection of PFC emulsion in the tumor. Note the enhancement of the center of the node (arrow) leaving the marginal sinus unenhanced (arrowheads). This was shown to be due to the accumulation of the agent in lymph node macrophages.

(a)

(b)